

EVOLUTIONARY AND ACCLIMATION-INDUCED VARIATION IN THE HEAT-SHOCK RESPONSES OF CONGENERIC MARINE SNAILS (GENUS *TEGULA*) FROM DIFFERENT THERMAL HABITATS: IMPLICATIONS FOR LIMITS OF THERMOTOLERANCE AND BIOGEOGRAPHY

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Summary

Heat stress sufficient to cause cellular damage triggers the heat-shock response, the enhanced expression of a group of molecular chaperones called heat-shock proteins (hsps). We compared the heat-shock responses of four species of marine snails of the genus *Tegula* that occupy thermal niches differing in absolute temperature and range of temperature. We examined the effects of short-term heat stress and thermal acclimation on the synthesis of hsps of size classes 90, 77, 70 and 38 kDa by measuring incorporation of ³⁵S-labeled methionine and cysteine into newly synthesized proteins in gill tissue. Temperatures at which enhanced synthesis of hsps first occurred (T_{on}), temperatures of maximal induction of hsp synthesis (T_{peak}) and temperatures at which hsp synthesis was heat-inactivated (T_{off}) were lowest in two low-intertidal species from the temperate zone, *T. brunnea* and *T. montereyi*, intermediate in a mid- to low-intertidal species of the temperate zone, *T. funebris*, and highest in a subtropical intertidal species from the Gulf of California,

T. rugosa. Synthesis of hsps and other classes of protein by *T. brunnea* and *T. montereyi* was heat-inactivated at temperatures commonly encountered by *T. funebris* during low tides on warm days. In turn, protein synthesis by *T. funebris* was blocked at the upper temperatures of the habitat of *T. rugosa*. Acclimation of snails to 13 °C, 18 °C and 23 °C shifted T_{on} and T_{peak} for certain hsps, but did not affect T_{off} . The heat-shock responses of field-acclimatized snails were generally reduced in comparison with those of laboratory-acclimated snails. Overall, despite the occurrence of acclimatory plasticity in their heat-shock responses, genetically fixed differences in T_{on} , T_{peak} and T_{off} appear to exist that reflect the separate evolutionary histories of these species and may play important roles in setting their thermal tolerance limits and, thereby, their biogeographic distribution patterns.

Key words: acclimation, biogeography, heat-shock protein, intertidal zone, protein synthesis, *Tegula* spp., thermotolerance.

Introduction

The heat-shock response has received an enormous amount of study, especially since the discovery that heat-shock proteins (hsps), which are also termed stress proteins, are a subset of the large family of molecular chaperones that function in all types of cell to ensure the proper folding and compartmentation of proteins (Feige et al., 1996; Gething, 1997; Bukau and Horwich, 1998). Because hsps prevent the aggregation of heat-damaged proteins and facilitate their renaturation following a heat shock, they are likely to play an important role in thermotolerance (Parsell and Lindquist, 1993, 1994). Despite the large literature related to hsps, surprisingly few studies have examined their expression under natural (field) temperature conditions or the variation in the heat-shock response between species, which may contribute to establishing differences in their thermal tolerance limits (for a review, see Feder and Hofmann, 1999).

Among the important and largely unanswered questions that need to be addressed are the following. What characteristics of the heat-shock response differ between species adapted to different thermal niches? How do such important characteristics as the onset temperature of enhanced synthesis of hsps (T_{on}), the temperature of maximal hsp expression (T_{peak}) and the upper thermal limit for synthesis of hsps (T_{off}) correspond to habitat temperatures? Which of these characteristics of the heat-shock response are genetically fixed, and which can be modified by acclimatization in the field or acclimation in the laboratory? How large a change in exposure temperature is required to elicit an acclimatory response? Over what range of temperatures can acclimation be induced? Do acclimatory responses differ between steno- and eurythermal species? Do the expression patterns of different size classes of hsp differ within a species? Does expression of different

classes of hsp differ among species? Answers to these questions may reveal how evolutionary variation in the heat-shock response adapts organisms to their thermal niches and contributes to biogeographic patterning.

To address the above questions, we have initiated studies of the heat-shock response in marine snails belonging to the genus *Tegula*. Because of their diverse latitudinal and vertical distribution patterns, congeners of *Tegula* occupy thermal niches that vary widely in absolute temperature and range of temperature (Riedman et al., 1981; Watanabe, 1984; Hellberg, 1998). In the present study, we examined four congeners from temperate and subtropical habitats. *Tegula brunnea* and *T. montereyi* are low-intertidal to subtidal cool-temperate species that seldom face exposure to air (emersion) and are unlikely to encounter temperatures in excess of approximately 20–25 °C. A mid- to low-intertidal-zone congener found at the same latitude, *T. funebris*, encounters much higher peak temperatures, 33 °C or more, when emersed. A fourth species, *T. rugosa*, is endemic to the rocky intertidal zone of the Gulf of California, where it encounters air and water temperatures in the region of 40 °C. Thus, these four congeners would be expected to possess widely different thermal tolerances and, we hypothesized, adaptive differences in their heat-shock responses. Another advantage afforded by these congeners is that any differences observed in the heat-shock responses are likely to indicate adaptation due to selective pressures arising from different thermal environments during their recent separate evolutionary histories rather than merely reflecting phylogenetic distance (Hellberg, 1998).

Using field-acclimatized and laboratory-acclimated specimens of *Tegula*, we determined the effects of acute thermal exposure on rates of incorporation of ³⁵S-labeled methionine and cysteine into proteins in gill tissue, which readily accumulates dissolved free amino acids (Wright, 1988). We quantified the amount of new synthesis of hsps belonging to four size classes, 90 kDa (hsp90), 77 kDa (hsp77), 70 kDa (hsp70) and 38 kDa (hsp38) relative to a non-heat-shocked control. Comparisons among the differently adapted,

acclimatized and acclimated snails suggest that, despite there being acclimatory plasticity in their heat-shock responses, the four congeners have genetically fixed differences in these responses and in their upper thermal limits of protein synthesis that are of importance in determining their distinct vertical and latitudinal distribution patterns.

Materials and methods

Organisms, distribution patterns and collection sites

The vertical distributions of the three temperate-zone *Tegula* species used in this study are given in Fig. 1. *Tegula funebris* (Adams) has the widest latitudinal range, from Vancouver Island, British Columbia, Canada (48°25'N), to central Baja California, Mexico (28°00'N) (Abbott and Haderlie, 1980; Hellberg, 1998). *Tegula brunnea* (Philippi) is found from Cape Arago, Oregon, USA (43°21'N), to the Channel Islands, California, USA (34°00'N). *Tegula montereyi* (Kiener) occurs from Sonoma County, California, USA (38°17'N), to the Channel Islands, USA (Abbott and Haderlie, 1980; Hellberg, 1998). The three temperate *Tegula* species were collected at Hopkins Marine Station (HMS) of Stanford University in Pacific Grove, California (36°36'N, 121°54'W). The mid-intertidal *T. rugosa* (Adams), which is endemic to the northern part of the Gulf of California (Hellberg, 1998), was collected in San Felipe, Baja California, Mexico (27°20'N, 106°00'W). Large adults were used exclusively in all experiments, and the sizes of specimens were similar in all four species.

Measurements of body temperatures in the field

We used gelatin-filled snail shells to record internal body temperatures under field conditions (100% gelatin, Nabisco Foods). During hardening of the gelatin, we inserted a thermistor (Yellow Springs Instruments, model 44006; accuracy ±0.2 °C) into the interior of the shell and subsequently covered the opening with silicone sealant. The extension of the thermistor was connected to a Stow Away XTI (Onset, Massachusetts, USA) temperature data logger

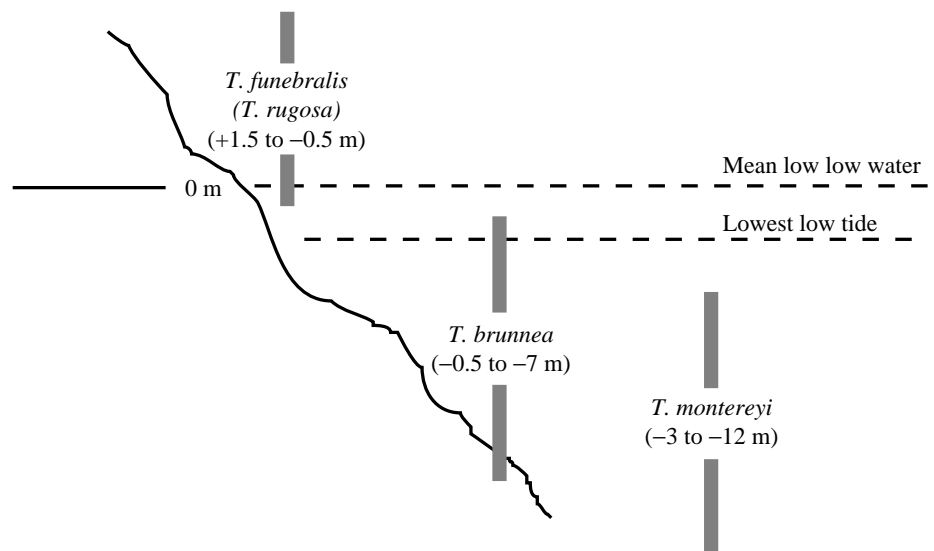


Fig. 1. Vertical distribution ranges of three temperate snail species of the genus *Tegula* (*T. funebris*, *T. brunnea* and *T. montereyi*) along the intertidal–subtidal transition at Hopkins Marine Life Refuge, Pacific Grove, California, USA (after Riedman et al., 1981; Watanabe, 1984). The subtropical *T. rugosa* has a vertical distribution range similar to that of *T. funebris*.

(temperature accuracy greater than $\pm 0.7^\circ\text{C}$), which was placed in a submersible case (Ikelite). The shells were then glued to rocks using an epoxy resin (A-788 Splash Zone Compound, Z-SPAR) near sites where *Tegula* is abundant during low tide, and temperatures were recorded continuously from 22 March to 17 April 1996. Temperature data were recorded for *T. brunnea* from a site 0.08 m below mean low low water (MLLW), and for *T. funebris* at a site 0.51 m above MLLW.

To determine how closely the temperatures of the gelatin-filled shells simulated those of live snails, we measured the body temperatures of live snails by inserting a thermistor as far as possible into the mantle cavity. Temperatures in the continuously monitored gelatin-filled shells differed by less than 1°C from temperatures determined during shorter-term measurements of live snails (L. Tomanek and G. N. Somero, unpublished data). Henceforth, we will refer to temperature measurements from gelatin-filled shells as 'body temperatures'.

Tidal data for Monterey Bay were obtained from the HMS website (www-marine.stanford.edu/HMSweb/Tides.txt), and sunrise and sunset times from a US Navy website (www.usno.navy.mil/). Average seawater temperature at 16 m depth approximately 300 m offshore from the Hopkins Marine Life Refuge was measured in spring 1996 using a Stow Away XTI data logger (unpublished data of Dr J. J. Leichter, Woods Hole Oceanographic Institution).

Thermal tolerance measurements

Two thermal tolerance studies were conducted. In the first, which was designed to measure differences among field-acclimatized specimens of the three temperate-zone congeners, specimens of *T. funebris*, *T. brunnea* and *T. montereyi* were collected in mid-August 1996 and placed in a seawater aquarium at 14°C . Within 48 h of collection, we assessed thermal tolerance by raising the incubation temperature by 1°C every 12 min, up to a maximal temperature of 44°C . When each target temperature was reached, 20 snails of each species were removed from the aquarium and immediately checked for survival by prodding the underside of the foot to determine whether a withdrawal reaction occurred.

The second thermal tolerance study was designed to allow comparison between whole-snail thermal tolerance and the thermal resistance of protein synthesis in gill tissue. Specimens of *T. funebris* and *T. brunnea* acclimated to 13°C for 32–44 days were exposed for 2.5 h to various temperatures ($N=20$ for each temperature) to mimic conditions used for the incubation of isolated gill tissues. Survival of snails was determined as described above by testing for the foot withdrawal response.

Thermal acclimation

Specimens of the three temperate-zone species were collected for the acclimation experiment in mid-July 1997 and either immediately used for radiolabeling experiments (field-acclimatized control group) or kept in temperature-controlled (13°C , 18°C and 23°C) circulating seawater aquaria for 30–34 days. *Tegula rugosa* was collected in mid-July 1998 and acclimated at 23°C for 30 days. Specimens were kept

constantly immersed and fed regularly with freshly collected giant kelp (*Macrocystis pyrifera*).

Heat-shock protocol and tissue preparation

We dissected gill tissue under non-heat-shock-inducing conditions (13°C sea water for temperate and 23°C sea water for subtropical species) and immediately placed the tissue into plastic microcentrifuge tubes containing filtered ($0.2\mu\text{m}$) sea water containing 10mmol^{-1} glucose. Tubes were pre-equilibrated at 13°C (23°C for *T. rugosa*) before the start of the experiment. Gill tissues were aerated every 30 min after dissection. The tubes containing gill tissue were then placed into different water baths preheated to the desired incubation temperature.

Samples of gill tissues were incubated at various temperatures for 2.5 h (see Figs 5–8). After incubation, tissues were placed at 13°C (23°C for *T. rugosa*) for 15 min before ^{35}S -labeled methionine/cysteine (NEN) was added to the tube. The duration of the thermal exposure was less than the full period of aerial exposure that snails in the mid-intertidal would typically experience during emersion. Thus, a 2.5 h exposure is conservative in terms of the severity of the heat stress that might occur under field conditions. Higher concentrations of labeled amino acids were used for *T. brunnea* and *T. montereyi* (12.21MBq ml^{-1} ; 30–45 mg wet mass) than for *T. funebris* and *T. rugosa* (8.14MBq ml^{-1} ; 15–25 mg) to compensate for higher tissue mass and lower uptake rates in the former two species. We then incubated the tubes for 4 h at 13°C (23°C for *T. rugosa*), a period adequate to allow protein synthesis to occur. After this incubation period, we washed the gill tissue in ice-cold sea water, added homogenization buffer [32mmol^{-1} Tris/HCl, pH 7.5 at 4°C , 2% (w/v) SDS, 1mmol^{-1} EDTA, 1mmol^{-1} Pefabloc (Boehringer Mannheim), $10\mu\text{g ml}^{-1}$ pepstatin and $10\mu\text{g ml}^{-1}$ leupeptin] to the tubes, and then froze the tubes on dry ice. The samples were then stored at -70°C . To prepare homogenates for autoradiography, the frozen samples were thawed in a dry bath for 5 min at 100°C and then homogenized with a silicone pestle. Homogenates were incubated at 100°C for 5 min, homogenized a second time and then centrifuged at $15\,800g$ for 15 min. The supernatant was removed and stored at -70°C . No proteolytic activity was detected during this homogenization procedure. To determine the amount of ^{35}S -labeled amino acids incorporated into newly synthesized proteins, we pipetted samples (5 or $10\mu\text{l}$) of gill supernatant onto GF-C glass-fiber filters (Whatman) and allowed them to dry in air. To remove unincorporated ^{35}S -labeled amino acids, we washed the filters once in ice-cold 10% trichloroacetic acid for 10 min, followed by two 5-min washes with 5% trichloroacetic acid at room temperature (approximately 20°C), and finally rinsed the filters in 95% ethanol. The filters were dried at room temperature, and counts per minute of incorporated ^{35}S -labeled amino acids were quantified in a scintillation counter.

Gel electrophoresis and fluorography

For most tissue samples, we loaded approximately

500×10^3 counts min^{-1} onto each lane of 10% SDS–polyacrylamide gels. The gels were run first at 25 mA for 80 min (stacking gel) and then at 30 mA for 150 min (resolving gel). We obtained significantly lower incorporation rates for gill tissue exposed to higher temperatures (33 °C for *T. brunnea* and *T. montereyi*, 36 °C, 38 °C and 39 °C for *T. funebris* and 40 °C and 42 °C for *T. rugosa*) and therefore loaded only 100×10^3 counts min^{-1} from these samples onto the gels to avoid over-loading of gel wells. Gels were stained overnight with Coomassie R-250 in 10% acetic acid and destained for 2 h in 10% acetic acid and 30% methanol. The gels were treated with EN³HANCE (NEN) for 1 h according to the manufacturer's instructions, and then dried and exposed to pre-flashed film (Kodak X-OMAT) at –70 °C for 8 h (500×10^3 counts min^{-1}) or 18 h (100×10^3 counts min^{-1}). We corrected for the fact that 500×10^3 counts min^{-1} samples emitted 2.23 times more counts over 8 h than did samples with 100×10^3 counts min^{-1} over 18 h. All levels of hsp expression are given after this correction, i.e. after normalization to 500×10^3 counts min^{-1} at 8 h exposure time.

Image analysis, quantification of expression of heat-shock proteins and statistical analyses

We scanned film images on a densitometer (Sharp JX-330) and analyzed the digitized images with image-analysis software (ImageMaster 1D, Version 2.01, Pharmacia) to quantify the amount of newly synthesized protein in each hsp size class. To determine T_{on} , T_{peak} and T_{off} , we developed a quantification protocol that used the intensity of the relevant hsp mass band at 13 °C, a temperature at which we incubated gill tissue of all specimens from all acclimatization and acclimation regimes (with the exception of *T. rugosa*, for which we used the band at 23 °C), as the index for

normalization. The intensities of this band at other temperatures were expressed relative to the intensity at 13 °C for all classes of hsp. We emphasize that all ³⁵S-incorporation experiments on the three temperate species were performed at a common incubation temperature (13 °C). Thus, no temperature effects on rates of protein synthesis are present in data from temperate species.

Note that hsps are named according to molecular mass, but have not been further characterized with respect to the number of isoforms present or homology with hsps of other species.

Comparisons of hsp band intensities were performed using a one-sided Dunnett test after a one-way analysis of variance (ANOVA; SYSTAT Software; Systat, Inc.). For the ANOVA, data were log-transformed; incubation temperature was used as the independent variable and expression level of hsps as the dependent variable. We describe the first temperature at which band intensity was significantly higher ($P \leq 0.05$) than the band intensity of the 13 °C control group (23 °C in case of *T. rugosa*) as the onset temperature (T_{on}) of the synthesis of a particular hsp. To reduce clutter in Figs 5–8, which present the digitized data, we have not indicated significant differences on the figures, but instead discuss these in the text.

Results

Vertical distributions, field temperature measurements and thermal tolerances

The three temperate species of *Tegula* examined in this study have distinct vertical distributions along the intertidal-to-subtidal transition at HMS (Fig. 1). *In situ* body temperatures of *Tegula* congeners reflect the vertical distribution ranges of the species and the effects of the tidal cycle. Fig. 2 displays a continuous series of temperature

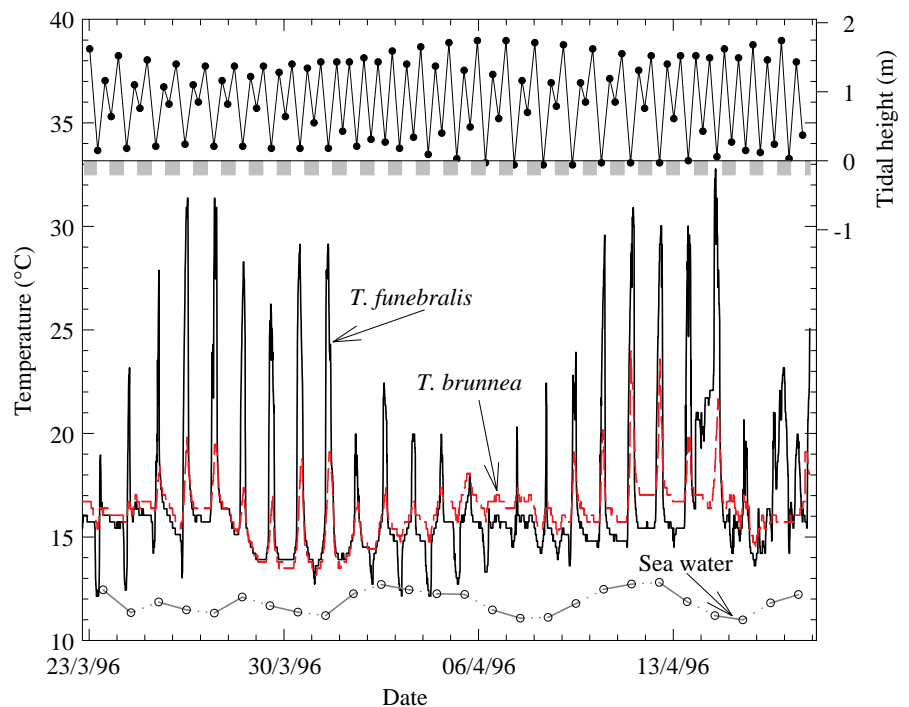


Fig. 2. Field body temperature measurements of gelatin-filled shells of *Tegula funebris* (black solid line) and *T. brunnea* (red broken line) from different intertidal heights (0.51 m above mean low water, MLLW, for *T. funebris* and 0.08 m below MLLW for *T. brunnea*) at Hopkins Marine Life Refuge, Pacific Grove, California. Tidal patterns and day (white) and night (gray) cycles are given for comparison in the upper graph. Seawater temperature measurements at 16 m depth are shown by the dotted line with open circles.

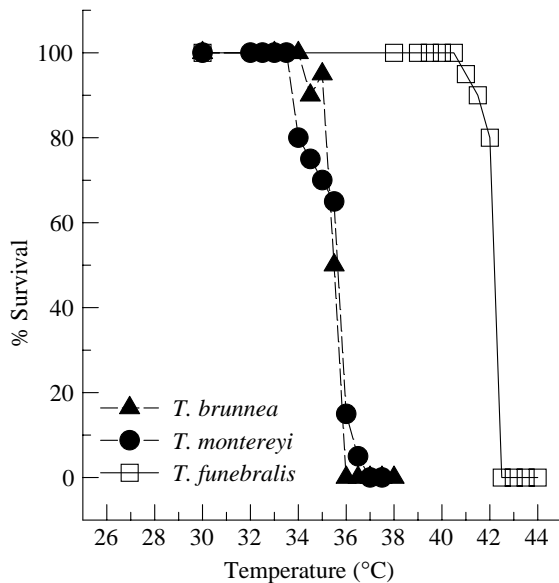


Fig. 3. Percentage survival of field-collected specimens (mid-August 1996) of three species of the genus *Tegula* (*T. funebris*, *T. brunnea* and *T. montereyi*) after exposure to increases in temperature. The rate of temperature increase was 1 °C every 12 min ($N=20$ for each data point).

measurements of gelatin-filled shells of *T. funebris* and *T. brunnea* obtained at intertidal sites within the vertical distribution range of each species. The vertical heights that were chosen (Fig. 2) represent upper thermal extremes for these two species at HMS. For *T. brunnea*, this height represents the extreme upper limit of its vertical distribution range. *Tegula funebris* does not move during emersion and frequently hides in or near crevices at its highest sites of occurrence. Thus, it experiences longer exposures to solar radiation and attains higher body temperatures in the center of the mid-intertidal zone (L. Tomanek and G. N. Somero, unpublished observations).

Body temperatures for both species varied with the daily tidal rhythm (Fig. 2), but maximal changes in body temperature during a tidal cycle were more than twice as great for *T. funebris* (approximately 19 °C) as for *T. brunnea* (approximately 7 °C). The maximal body temperatures measured for *T. funebris* (approximately 33 °C) are approximately 10 °C higher than those for *T. brunnea* (approximately 24 °C). *Tegula funebris* frequently experienced temperatures between 27 °C and 33 °C (Fig. 2), but *T. brunnea* experienced temperatures as high as 21 °C only during three extreme midday low tides (11, 12 and 14 April; Fig. 2).

We also observed longer-term periodicity linked to the timing of low tides. For example, the series of high body temperatures observed during daytime low tides for *T. funebris* during the last week of March (Fig. 2) was followed by an 8–10 day period of low peak body temperatures in early April, when low tides occurred between the early evening and early morning hours.

In general, we assume that the temperatures determined or estimated for *T. brunnea* approximate those that *T. montereyi* experiences, because both species remain immersed most of the time. Although we do not have field body temperature measurements for *T. rugosa*, summer water temperatures at the collection sites near San Felipe, Baja California, reach at least 36 °C (Dietz and Somero, 1992), and snails exposed during daytime low tides are apt to be even warmer.

To determine whether the differences in vertical distribution and field body temperature of the three temperate-zone congeners were associated with differences in heat tolerance, we assessed the survival of field-acclimatized *T. funebris*, *T. brunnea* and *T. montereyi* exposed to an increase in seawater temperature of 1 °C every 12 min (Fig. 3). Under this heating regimen, the temperature of 50% mortality (LT_{50}) was 42.5 °C for *T. funebris* and 36.0 °C for *T. brunnea* and *T. montereyi*.

Effect of acclimation

Interspecific differences

The autoradiographs in Fig. 4 show the patterns of protein synthesis, at a common incubation temperature of 13 °C, following exposure of isolated gills from 13 °C- and 23 °C-acclimated *T. funebris* and *T. brunnea* to several temperatures. We used densitometric analysis of such autoradiographs to generate Figs 5–8, which show the relative levels of synthesis of hsp70, hsp38, hsp90 and hsp77, respectively. Data are shown for 23 °C-acclimated individuals of all four species, as well as for the 13 °C- and 18 °C-acclimated and summer field-acclimatized specimens of the three temperate-zone species.

Three characteristic interspecific differences were observed in most comparisons. First, T_{on} varied positively with habitat (adaptation) temperature. For example, comparisons of 13 °C-acclimated *T. brunnea* and *T. funebris* (Figs 4, 5) show that T_{on} of hsp70 occurred at 24 °C in *T. brunnea* and at 27 °C in *T. funebris*. For hsp90, comparisons of the four species (23 °C acclimation groups) show a T_{on} of 24 °C for *T. brunnea* and *T. montereyi*, of 27 °C for *T. funebris* and of 30 °C for *T. rugosa* (Fig. 7).

Second, T_{peak} showed a positive trend with habitat temperature for hsp70 for all species over all acclimation temperatures (Fig. 5). This trend also was found for hsp38 (Fig. 6) and hsp77 (Fig. 8), but not consistently for the expression of hsp90 (Fig. 7). Third, among all classes of hsp, a positive trend was found between adaptation temperature and T_{off} . This trend was also evident for the thermal limits of protein synthesis *per se* (Figs 5–9). The values for T_{off} were near the upper limits of the thermal tolerance ranges for *T. funebris* and *T. brunnea* (Figs 9, 10). This is shown by the organismal heat tolerance data in Fig. 10, which were obtained using an incubation temperature regimen similar to that employed in the protein synthesis experiments. In addition, the difference between temperate intertidal and subtidal species in T_{off} (39 °C versus 33 °C) was similar to the interspecific differences in survival temperature, 6.5 °C, obtained using the protocol shown in Fig. 3. Furthermore, in *T. brunnea* and *T.*

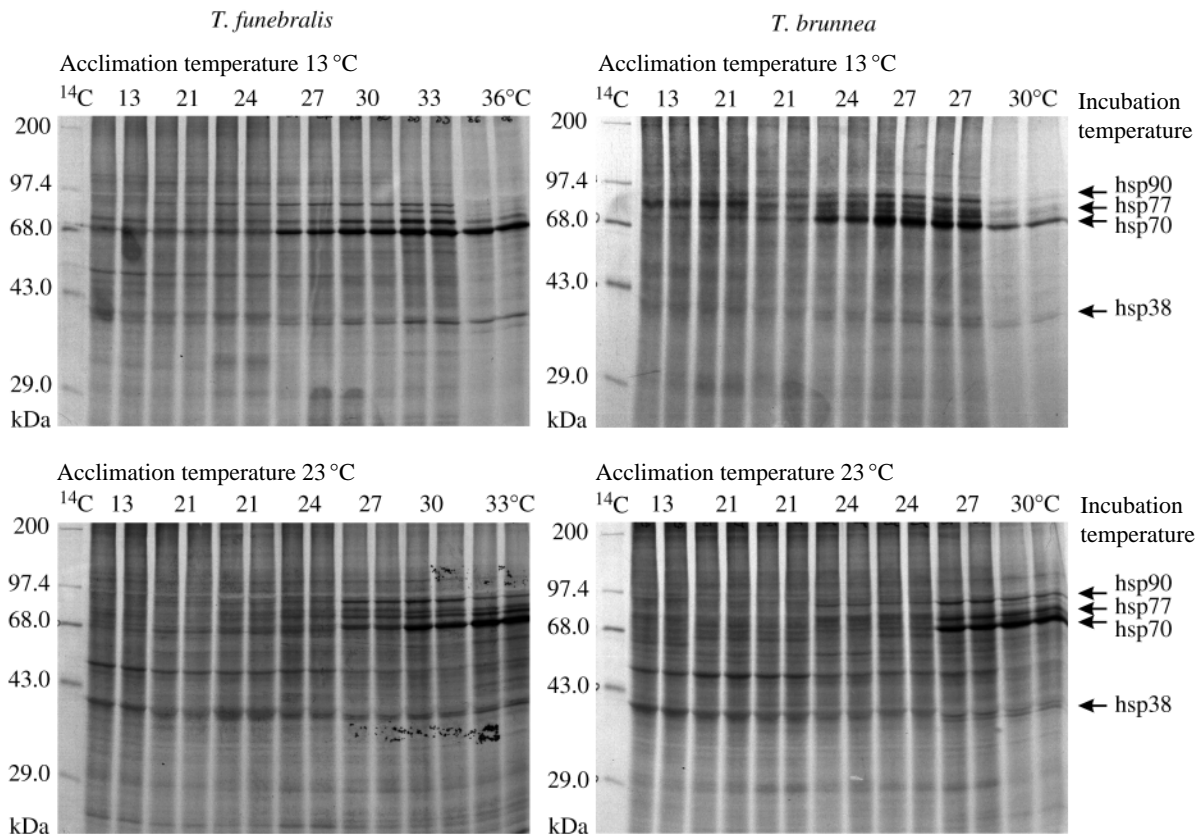


Fig. 4. Autoradiographs showing the effects of incubation temperature on incorporation of ^{35}S -labeled methionine/cysteine into proteins of gill tissue of *Tegula funebralis* and *T. brunnea* acclimated to either 13 or 23 °C. For each incubation temperature, duplicates of a gill sample from a single snail were loaded on a 10% SDS-polyacrylamide gel. ^{14}C molecular mass markers are shown in the far left lane of each autoradiograph. All lanes were loaded with 500×10^3 counts min^{-1} and exposed for 8 h to pre-flashed X-ray film.

montereyi, protein synthesis was blocked at temperatures (30–33 °C) that were frequently experienced by *T. funebralis* during the spring (Fig. 2). In turn, protein synthesis in *T. funebralis* ceased at temperatures below the maximal body temperatures likely to be encountered by the subtropical intertidal species *T. rugosa*.

Differences among the four species were also noted in the relative intensities of induction of the different size classes of hsp. For example, for hsp90, comparisons among the 23 °C-acclimated snails showed that *T. brunnea* and *T. montereyi* increased synthesis most, and *T. rugosa* increased synthesis least (Fig. 7). In contrast, the increase in synthesis of hsp38 was greatest in *T. rugosa* and *T. funebralis* and smallest in *T. brunnea* and *T. montereyi* (Fig. 6). For hsp77, relative synthesis was greatest in *T. funebralis* (Fig. 8).

Specific expression patterns of heat-shock proteins

To examine the phenotypic plasticity of the heat-shock response, we acclimated specimens of the three temperate-zone species to 13 °C, 18 °C and 23 °C under constant submersion. These temperatures lie within the range routinely encountered by *T. funebralis*, whereas 23 °C is near the upper limit of the temperature ranges of the lower-occurring species *T. brunnea* and *T. montereyi*.

For *T. brunnea* and *T. montereyi*, an increase in acclimation temperature from 13 to 23 °C led to an increase in T_{on} of hsp70 from 24 to 27 °C (Figs 4, 5). The temperature of maximal induction (T_{peak}) of hsp70 in these two species shifted from 27 to 30 °C as acclimation temperature rose from 13 to 18 °C (Fig. 5). No additional increase in T_{peak} was seen following acclimation to 23 °C. *Tegula funebralis* showed no acclimation-induced changes in T_{on} or T_{off} for hsp70 (Figs 4, 5). However, T_{peak} of hsp70 increased from 33 to 36 °C as acclimation temperature increased from 13 to 18 °C. As in the case of *T. brunnea* and *T. montereyi*, no additional changes were observed when the acclimation temperature was increased from 18 to 23 °C (Fig. 5).

For hsp38, acclimation had a marked effect on the responses of *T. brunnea* and *T. montereyi*. Although 13 °C-acclimated specimens of both species exhibited two- to threefold increases in synthesis of hsp38 at 27 °C, induction of hsp38 synthesis in the 18 °C- and 23 °C-acclimated specimens was much less intense (Fig. 6). *Tegula funebralis* showed enhanced synthesis of hsp38 at temperatures above 30 °C in all acclimation groups. For hsp90, *T. funebralis* exhibited a shift in T_{on} and a consistent attenuation of the maximal level of hsp90 synthesis with an increase in acclimation temperature from 13 to 18 °C, but *T. brunnea* and *T. montereyi* did not show these effects (Fig. 7).

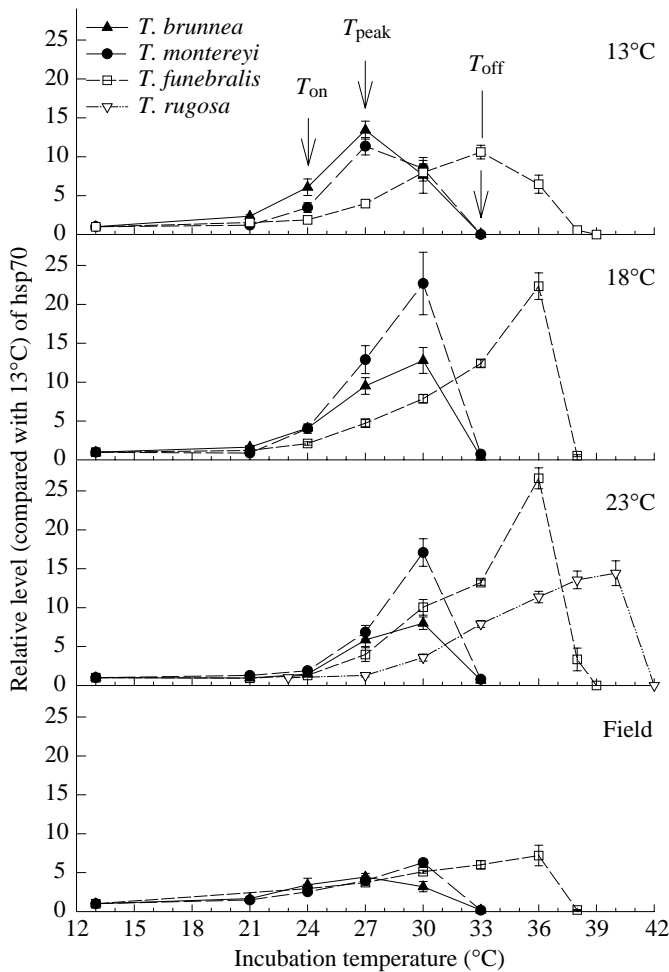


Fig. 5. Relative induction (compared with the 13 °C control group) of hsp70 in the three temperate *Tegula* congeners *T. funebris*, *T. brunnea* and *T. montereyi* after laboratory acclimation at 13 °C, 18 °C and 23 °C for 30–34 days and field acclimatization (July 1997). Data are also given for 23 °C-acclimated *T. rugosa* (23 °C control group). T_{on} indicates the onset temperature, T_{peak} the temperature of maximal induction and T_{off} the cessation temperature of hsp synthesis. Values are means \pm 1 S.E.M. $N=5$ for all data points except for 13 °C-acclimated *T. funebris* at 36 °C ($N=4$), for 23 °C-acclimated *T. funebris* at 33 °C and *T. brunnea* at 13 °C ($N=4$) and for field-acclimatized *T. funebris* at 27 °C and *T. montereyi* at 13 °C and 30 °C ($N=4$).

An increase in acclimation temperature from 18 to 23 °C did not change hsp expression patterns in any species. For hsp77, the responses of *T. brunnea* and *T. montereyi* were little affected by acclimation, whereas *T. funebris* showed a large increase in intensity of response at the two higher acclimation temperatures (Fig. 8).

In general, acclimatory changes in T_{on} and T_{peak} occurred with a shift in acclimation temperature from 13 to 18 °C, but only minor additional changes were elicited by a further increase in acclimation temperature to 23 °C. Unlike T_{on} and T_{peak} , T_{off} showed no response to acclimation (Figs 5–9).

At higher heat-shock-inducing temperatures, synthesis of proteins other than hsp was typically much reduced,

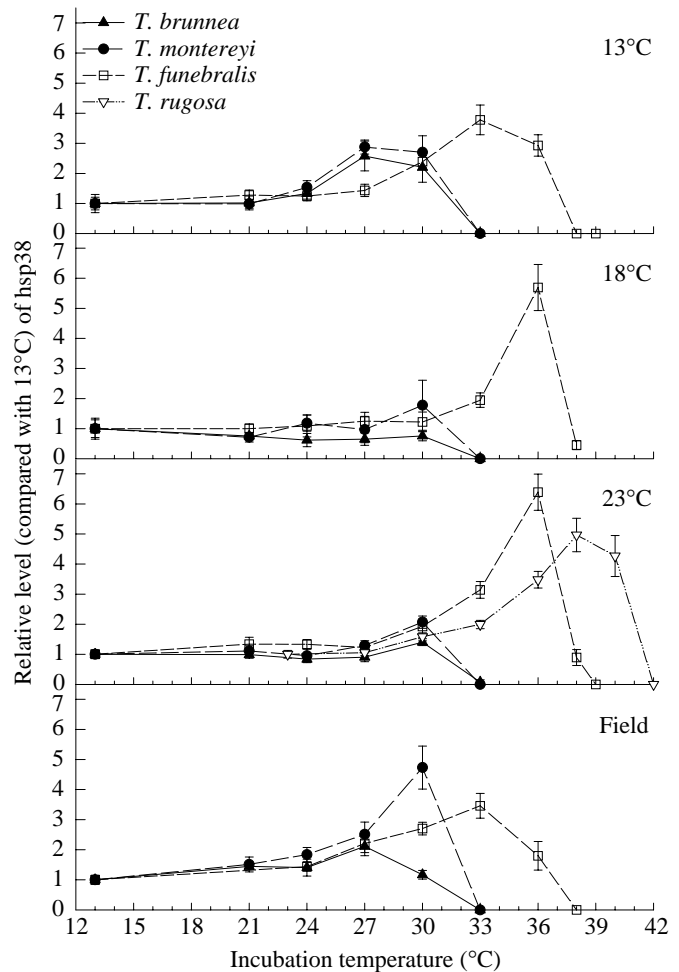


Fig. 6. Relative induction (compared with the 13 °C control group) of hsp38 in the three temperate *Tegula* congeners *T. funebris*, *T. brunnea* and *T. montereyi* after laboratory acclimation at 13 °C, 18 °C and 23 °C for 30–34 days and field acclimatization (July 1997). Data are also given for 23 °C-acclimated *T. rugosa* (23 °C control group). Values are means \pm 1 S.E.M. $N=5$ for all data points, except for 13 °C-acclimated *T. funebris* at 36 °C ($N=4$), for 18 °C-acclimated *T. montereyi* at 13 °C ($N=4$), 21 °C ($N=4$), 24 °C ($N=4$), 27 °C ($N=4$) and 30 °C ($N=4$), for 23 °C-acclimated *T. funebris* at 33 °C ($N=4$) and for field-acclimatized *T. funebris* at 27 °C ($N=4$) and *T. montereyi* at 13 °C ($N=4$) and 30 °C ($N=4$).

especially in 13 °C-acclimated snails (Figs 4, 9). However, in all three temperate-zone species, increasing acclimation temperatures restored to some degree the synthesis of non-hsps at higher temperatures, e.g. at 30 °C in *T. brunnea* and *T. montereyi* (Fig. 9). Thus, acclimation to higher temperatures allowed synthesis of non-hsps to occur at higher levels at the maximal temperatures at which synthesis was possible, even though T_{off} values were not altered by acclimation. Synthesis of hsp was also increased at the highest temperatures at which synthesis was possible. In some cases, the maximal levels of hsp synthesis occurred at higher temperatures (Figs 4–9).

Variation was also observed among size classes of hsp

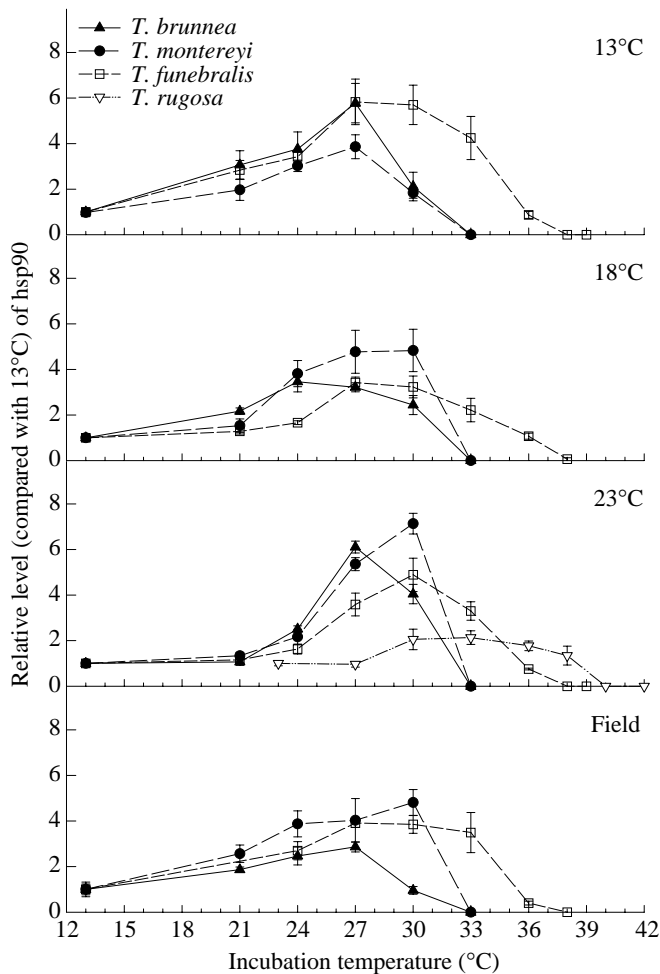


Fig. 7. Relative induction (compared with the 13 °C control group) of hsp90 in the three temperate *Tegula* congeners *T. funebris*, *T. brunnea* and *T. montereyi* after laboratory acclimation at 13 °C, 18 °C and 23 °C for 30–34 days and field acclimatization (July 1997). Data are also given for 23 °C-acclimated *T. rugosa* (23 °C control group). Values are means \pm 1 S.E.M. $N=5$ for all data points, except for 13 °C-acclimated *T. funebris* at 36 °C ($N=4$), for 23 °C-acclimated *T. funebris* at 33 °C ($N=4$) and for field-acclimatized *T. funebris* at 27 °C and 33 °C and *T. montereyi* at 13 °C ($N=4$).

within a species at a common acclimation temperature. For example, 13 °C-acclimated *T. funebris* induced hsp90 (Fig. 7) at 24 °C, hsp70 (Fig. 5) at 27 °C and hsp38 (Fig. 6) and hsp77 (Fig. 8) at 30 °C. *T. brunnea* and *T. montereyi* acclimated to 13 °C induced hsp70 and hsp90 at 24 °C, but hsp38 and hsp77 at 27 °C. In general, hsps were induced in the following order (lowest to highest induction temperatures): hsp90 < hsp70 < hsp77 < hsp38 (Fig. 4).

Field-acclimatized specimens versus laboratory-acclimated specimens

As shown in Figs 5–8, the heat-shock responses of field-acclimatized snails both resembled and differed from those of the laboratory-acclimated conspecifics. The T_{off} values were the same in the field-acclimatized and laboratory-acclimated

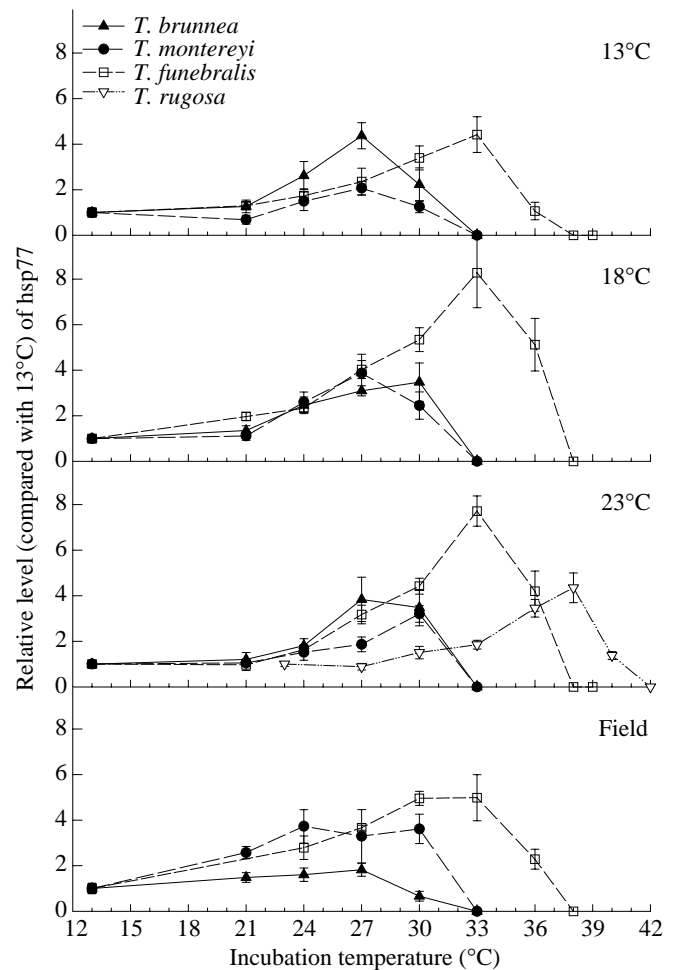


Fig. 8. Relative induction (compared with the 13 °C control group) of hsp77 in the three temperate *Tegula* congeners *T. funebris*, *T. brunnea* and *T. montereyi* after laboratory acclimation at 13 °C, 18 °C and 23 °C for 30–34 days and field acclimatization (July 1997). Data are also given for 23 °C-acclimated *T. rugosa* (23 °C control group). Values are means \pm 1 S.E.M. $N=5$ for all data points, except for 13 °C-acclimated *T. funebris* at 36 °C ($N=4$), for 23 °C-acclimated *T. funebris* at 36 °C ($N=4$) and for field-acclimatized *T. funebris* at 27 °C ($N=4$) and *T. montereyi* at 13 °C and 30 °C ($N=4$).

snails, which is further evidence for a genetically fixed upper thermal limit to protein synthesis in these species.

The most striking difference was found for hsp70. For all three temperate species, field-acclimatized specimens appeared to induce hsp70 only approximately one-quarter to one-third as strongly as snails from the 13 °C and 18 °C acclimation treatments (Fig. 5). However, this apparently lower intensity of induction in field-acclimatized snails is, in large measure, a consequence of the normalization procedure (see Materials and methods). Absolute levels of hsp70 synthesis were in fact higher in field-acclimatized specimens at all treatment temperatures (data not shown). This observation indicates that normalization to a common treatment temperature (13 °C in the present experiment) provides a means for evaluating how the heat-shock response varies among treatment temperatures, but

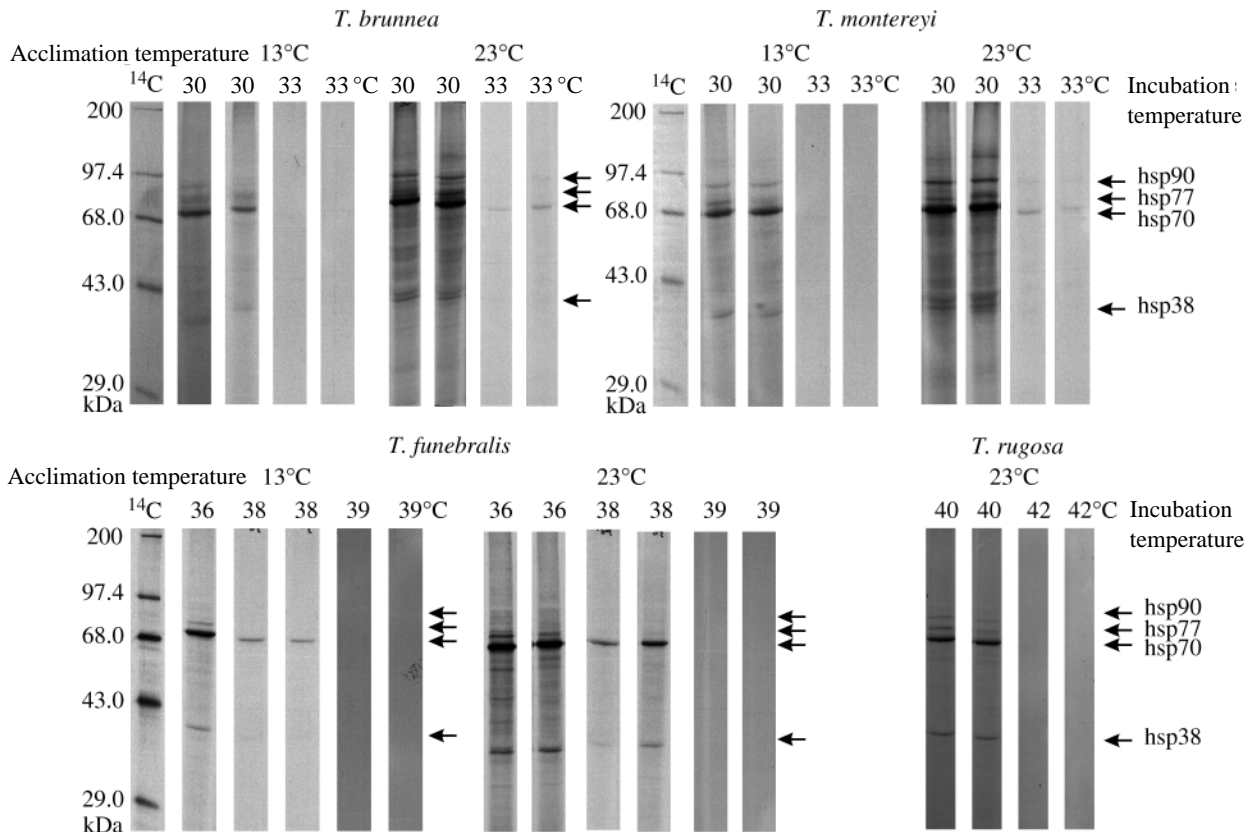


Fig. 9. Expression of newly synthesized proteins in specimens of *Tegula* congeners acclimated to different temperatures (13 °C and 23 °C). Arrows indicate the major hsps of size classes 90, 77, 70 and 38 kDa. Each lane of a 10% SDS–polyacrylamide gel was loaded with 100×10^3 counts min^{-1} . Pre-flashed X-ray film was exposed to the gel for 18 h for all temperature incubations except for the 30 °C lanes in *T. brunnea* and *T. montereyi*, which were loaded with 500×10^3 counts min^{-1} and exposed for 8 h to pre-flashed film. ¹⁴C molecular mass markers are shown in the far left lane of the autoradiographs for *T. brunnea*, *T. montereyi* and *T. funebris*.

it does not provide an index of the absolute level of synthesis that occurs in different acclimation or acclimatization groups (see Discussion).

Discussion

Biogeographic and ecological implications of interspecific variation in the heat-shock responses of Tegula congeners

Comparisons of heat-shock responses of four congeners of *Tegula* suggest that, despite some acclimatory plasticity in this response, genetically fixed differences exist between species in T_{on} , T_{peak} and T_{off} (Fig. 5) as well as in the upper thermal limits of protein synthesis in general. In the light of the thermal features of the distribution ranges of these species, each of these interspecific differences has implications for biogeographic patterning and for the *in situ* function of the heat-shock response.

First, the frequency with which the heat-shock response is induced *in situ* is likely to vary considerably between the four species. In their natural habitats, *T. brunnea* and *T. montereyi* are much less likely to experience temperatures that elicit enhanced expression of hsps than are *T. funebris* and *T. rugosa*. For example, during the 26 day period of midday low

tides between late March and mid-April, 1996 (Fig. 2), on at least 11 days body temperatures of *T. funebris* may have exceeded 27 °C, a temperature above or at the T_{on} of certain hsps, e.g. hsp70 and hsp90 (in 13 °C-acclimated specimens, Figs 5, 7). During this same 26 day period, on only a single day did the body temperature of *T. brunnea* reach a value as high as 24 °C, which is the T_{on} for hsp70 and hsp90 synthesis in 13 °C-acclimated specimens. Since water temperatures in the shallow intertidal habitat of *T. rugosa* rise to at least 36 °C in summer (Dietz and Somero, 1992), this intertidal snail must also commonly activate the heat-shock response *in situ*.

The heat-shock response is energetically costly (Sanchez et al., 1992; Heckathorn et al., 1996), so interspecific differences in the frequency with which it is induced could be associated with significant differences among these species in how temperature affects their energy budgets. Energy is required for the synthesis of hsps, and the chaperoning activity of most hsps requires hydrolysis of ATP. Over-expression of hsps can significantly decrease fitness (Feder et al., 1992; Krebs and Loeschcke, 1994), possibly as a consequence of the energy costs associated with the heat-shock response and the preferential synthesis of hsps, at the expense of synthesis of other types of proteins, at elevated temperatures (Figs 4, 9).

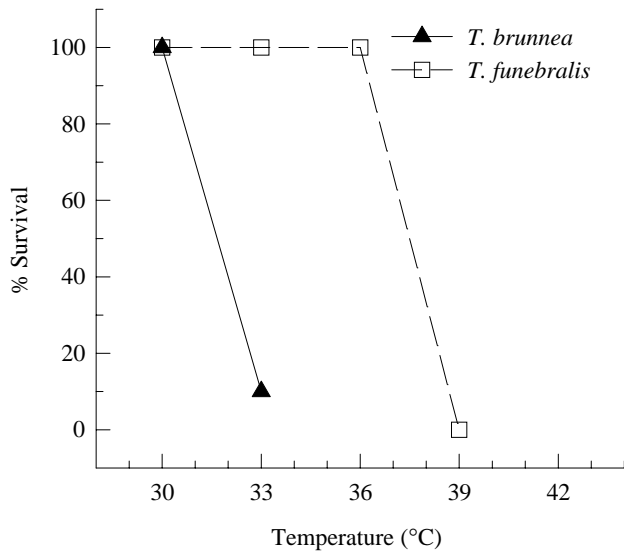


Fig. 10. Survival of 13°C-acclimated (5–7 weeks) *Tegula funebralis* and *T. brunnea* after exposure for 2.5 h at different temperatures ($N=20$ for each time point). Survival was assessed within 30 min of exposure by examining the withdrawal response of the foot.

Therefore, the species of *Tegula* that inhabit the mid-intertidal zone may face substantially higher energy costs related to synthesis of hsp and more frequent interruption of synthesis of non-hsp proteins than their lower-occurring congeners. The costs entailed in activating the heat-shock response may contribute to the upper limits of vertical distribution of intertidal invertebrates. A correlation between the induction of hsp at habitat temperatures and the variability of the thermal habitat has also been reported in a comparison of several species of *Hydra*, a freshwater cnidarian (Bosch et al., 1988, 1991; Gellner et al., 1992).

A second major difference found among the four congeners of *Tegula* is the upper thermal limit for the synthesis of hsp and other proteins. Protein synthesis of the two temperate species occurring lowest in the intertidal zone, *T. brunnea* and *T. montereyi*, was heat-inactivated at temperatures that are commonly experienced in the field by *T. funebralis* (Fig. 2) and at which the synthesis of some hsp, for instance hsp70 (Fig. 5), is maximal in this species. The thermal sensitivity of protein synthesis by *T. brunnea* and *T. montereyi* may prevent these two species from occurring in the mid-intertidal region inhabited by *T. funebralis*. Thermotolerance measurements (Fig. 10) certainly suggest that *T. brunnea* would not survive exposures between 30 and 33°C (over 2.5 h) that we frequently observed for *T. funebralis*. In turn, protein synthesis by *T. funebralis* ceased by 39°C, a temperature at which hsp synthesis was maximal in the most warm-adapted species, *T. rugosa*. The observation that thermotolerance of protein synthesis could not be modified through acclimation suggests a fixed genetic basis for these differences (Figs 5–9).

Further evidence that the heat sensitivity of protein synthesis may play a role in establishing thermal tolerance limits

and, thereby, contribute to biogeographic patterning along latitudinal and vertical gradients, is found in other studies of ectothermic animals with different distribution patterns. For example, a study of limpets of the genus *Collisella* demonstrated a 2°C difference in T_{off} between mid- and high-intertidal species (Sanders et al., 1991). Comparisons of a heat-adapted ant (genus *Cataglyphis*) from the Sahara with an ant living in temperate conditions (genus *Formica*) revealed a 6°C higher T_{off} in the former species (Gehring and Wehner, 1995). Protein synthesis in a more northern-occurring congener of *Mytilus*, *M. trossulus*, is heat-inactivated at temperatures near 30°C, whereas synthesis in the more southern-occurring species, *M. galloprovincialis*, continues at this temperature (Hofmann and Somero, 1996). These observations, taken in conjunction with the differences observed among *Tegula* congeners, provide evidence that differences in T_{off} may play an important role in establishing the biogeographic distributions of ectotherms.

Plasticity in the heat-shock response: laboratory acclimation and field acclimatization

We noted two types of plasticity in the heat-shock responses of differently acclimated and acclimatized conspecifics. First, we observed variation in the thermal responses of a given size class of hsp in differently acclimated or acclimatized individuals, which has also been observed in other species, including eurythermal goby fishes (*Gillichthys* spp.; Dietz and Somero, 1992) and intertidal mussels (*Mytilus* spp.; Roberts et al., 1997). Second, we found differences in response among size classes of hsp within a particular treatment group. The bases of the differences in relative amounts of synthesis and induction temperatures are undoubtedly complex, and the differences are probably based on the different roles played by the various classes of hsp (Parsek et al., 1993). For instance, although hsp90 is synthesized during heat stress, it does not seem to prevent heat inactivation of proteins under *in vivo* conditions (Nathan et al., 1997). *In vivo*, its contribution to the heat-shock response seems to be through an involvement in several signal-transducing pathways (Nathan et al., 1997; Pratt, 1998). Thus, more needs to be learned about the specific functions of the different classes of hsp in the heat-shock response before we can fully understand the importance of the inter- and intraspecific differences observed in this and other studies.

Although considerable acclimation-induced plasticity in the heat-shock response was noted, it did not usually occur across the full range of acclimation temperatures. Acclimatory effects occurred as acclimation temperature increased from 13 to 18°C, but a further increase in acclimation temperature from 18 to 23°C led to no major additional changes. The finding that neither *T. brunnea* nor *T. montereyi* showed additional acclimation effects when the temperature was raised from 18 to 23°C may reflect the infrequency with which these two species encounter temperatures as high as 23°C (Fig. 2). However, *T. funebralis*, which frequently encounters seawater temperatures above 23°C at its southern distribution limit, also

showed no additional acclimatory changes when temperature was increased from 18 to 23 °C. Thus, for *T. funebris*, the range of temperatures over which acclimation effects were observed was narrower than the range of body temperatures the organisms encounter in their habitats. Studies of acclimatory effects on mitochondrial function in congeners of abalone (genus *Haliotis*) also found that the range of temperatures over which acclimatory changes occurred was narrower than the range of habitat temperatures (Dahlhoff and Somero, 1993).

Despite the variation in T_{on} of hsp synthesis due to acclimatory history, the fact that interspecific differences in T_{on} were retained whatever the temperature of acclimation suggests that fixed genetic differences are important in setting the temperatures of induction of hsp synthesis. These genetically based differences could be due to gene regulatory factors that establish the set points for induction of hsp synthesis (Craig and Gross, 1991; Morimoto, 1998; Morimoto and Santoro, 1998) or to interspecific variations in the thermal stability of cellular proteins (Somero, 1995).

One additional effect of acclimation merits emphasis, the increased ability of gills from the most warm-acclimated specimens to synthesize proteins at high temperatures (Fig. 9). The synthesis of hsps during heat shock blocks the synthesis of non-hsps in some organisms because of the preferential translation of *hsp70* mRNA (Lindquist, 1980, 1981, 1993; Storti et al., 1980). A mild heat shock that induces hsp synthesis can prevent the inhibition of non-hsp synthesis during subsequent exposures to heat, indicating that pretreatment may affect the thermotolerance of translation (Petersen and Mitchell, 1981) by stabilizing translational initiation and/or chain elongation during heat shock (Beck and De Maio, 1994). Our observations therefore indicate that acclimation to higher temperatures, as well as a previous acute sub-lethal heat shock (Petersen and Mitchell, 1981), can lead to an increased ability to synthesize proteins at high temperatures. The mechanisms that allow protein synthesis to occur at higher rates near the maximal temperatures at which protein synthesis is possible appear to have no effect on T_{off} itself, which was unchanged by acclimation.

The influence of experimental design on the observed properties of the heat-shock response

Several results of this study illustrate how experimental design can influence the observed properties of the heat-shock response. First, the differences that were noted between summer field-acclimatized snails and laboratory-acclimated conspecifics provide a caveat about extrapolation from laboratory acclimation studies to the responses occurring in the field. Roberts et al. (1997), in a study of field-acclimatized and laboratory-acclimated mussels (*Mytilus californianus*), also found a greatly attenuated *hsp70* response in summer-acclimatized mussels. These observed differences between field- and laboratory-based studies suggest that the complex set of abiotic factors other than temperature that are present in field settings, such as nutritional status, desiccation stress, oxygen

availability and ultraviolet radiation, may alter the heat-shock response in ways that have not been simulated in laboratory acclimation studies.

Second, and following from the preceding observation, the normalization procedure used to gauge the amounts of newly synthesized hsps can affect the conclusions. If constitutive synthesis of a particular class of hsp is high, as in the case of field-acclimatized snails incubated at 13 °C, then the intensity of induction of hsps at hsp-inducing temperatures may be underestimated.

In conclusion, although several characteristics of the heat-shock responses in *Tegula* congeners responded to laboratory acclimation or field acclimatization, we interpret the consistent correlation between three key characteristics of the heat-shock response, T_{on} , T_{peak} and T_{off} , and normal habitat temperatures as a manifestation of genetically based interspecific differences that may play important roles in setting the latitudinal and vertical distribution limits of these species. Furthermore, the findings that protein synthesis is heat-inactivated at temperatures only slightly above the highest body temperatures measured and that the costly heat-shock response is frequently induced in higher-occurring intertidal species suggest that these species may currently be living near the upper extremes of their thermal tolerance ranges. This may put such species at a higher risk from global warming.

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